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M. Tenniswood

PI - Signature

Date

Table of Contents

Cover

SF 298

Foreword

Table of Contents

Body

1-9

Annual Report for Grant Number DAMD 17-97-7298

Covering Period September 1, 1998 to August 31, 1999

Title: Apoptosis and Tumor Invasion in Breast Cancer

Revised Statement of Work

Specific Aim 1: Characterize the ability of anti-estrogens to induce apoptosis and an invasive phenotype in a sub-population of cells in vitro

Task 1 (months 1-4): Develop time and dose dependence of acquisition of invasive phenotype of parental MCF-7 cell line [status: completed]

Task 2 (months 12-18): Characterize changes in gene expression and DNA fragmentation associated with the acquisition of the invasive phenotype in parental MCF-7 cells. [status: nearly completed]. RNA and protein have been extracted from the parental cell line, and the level of several transcripts, including clusterin, cathepsin B and uPA have been assessed by RT-PCR. The protein levels have been assessed by Western analysis. DNA fragmentation remains to be assessed by gel electrophoresis and by TUNEL staining. Immunofluorescence remains to be completed. **Estimated revised time frame: These experiments should be completed between months 20-30.**

Task 3 (months 2-8): Develop clonal MCF-7^{ae}_{inv} sublines of invasive cells. [status: completed]. We have isolated and clonally selected a number of MCF-7^{ae}_{inv} sublines, and have demonstrated that some, but not all, of the cell lines have retained sensitivity to ICI 182,780.

Task 4 (months 8-12): Characterize changes in gene expression associated with stable acquisition of invasive phenotype in MCF-7^{ae}_{inv} sublines. [status: partially complete]. We have isolated RNA and protein from several of the MCF-7^{ae}_{inv} sublines and level, and we are now performing RT-PCR and Western analyses to establish the changes in the levels of the relevant transcripts and proteins in the invasive sublines. **Estimated revised time frame: These experiments should be completed between months 20-30.**

Task 5 (months 12-15): Characterize the sensitivity of MCF-7^{ae}_{inv} to TNF α and anti-estrogens (tamoxifen, 4-hydroxytamoxifen and ICI 182,780) [status: partially complete]. We are comparing the sensitivity of these cell lines to other anti-estrogens (tamoxifen and 4-hydroxytamoxifen) to identify cell lines that have retained their sensitivity to anti-estrogens and others that have developed resistance to the drugs. There appear to be several different sensitivity profiles, and we are still cataloguing these profiles to determine whether there is an underlying biological explanation for

these differences that can be linked to estrogen receptor levels or the ability of the cells to initiate cell death. **Estimated revised time frame: These experiments should be completed between months 30-36.**

Specific Aim 2: Compare the sensitivity of the MCF-7^{ae}_{inv} sublines to other agents that induce apoptosis and determine if multiple agents can act synergistically to induce apoptosis

Task 1 (months 12-18): Characterize the effects of EB1089 and onapristone on apoptosis and invasion in parental MCF-7 cells compared to MCF-7^{ae}_{inv} sublines. [Status: recently initiated]. Very preliminary results, that need to be repeated have suggested that most MCF-7^{ae}_{inv} sublines are still sensitive to the synthetic vitamin D analog EB1089. **Estimated revised time frame: These experiments should be completed between months 24-30.**

Task 2 (months 12-18): Characterize the effects of simultaneous administration of anti-estrogen and EB1089 or onapristone on apoptosis and acquisition of the invasive phenotype on parental MCF-7 cells. Characterize the changes in gene expression and DNA fragmentation. [Status: not yet initiated]. **Estimated revised time frame: These experiments should be completed between months 30-36.**

Task 3 (months 15-24): Develop clonal MCF-7^{ona}_{inv} and MCF-7^{D3}_{inv} and compare their sensitivity to anti-estrogens and TNF α . **Estimated revised time frame: These experiments should be completed between months 33-36.**

Task 4 (months 12-24): Determine degree of cross sensitivity for each invasive cell type and characterize changes in gene expression. **Estimated revised time frame: These experiments should be completed between months 33-36.**
Task 4 cannot be initiated before task 3 has been completed.

As we have pointed out previously, prior to the initiation of the research we were asked to describe how we would pursue this work in vivo as part of a follow up study. At that time we added specific aim 3 to the research program, with the understanding that this specific aim was outside the scope of the two year funding period.

Specific Aim 3: To determine whether treatment with anti-estrogens or other apoptosis inducing reagents (such as vitamin D analogs) alters the rate and/or sites of metastasis and whether invasive cells generated by treatment with one reagent are sensitive to treatment with other reagents that induce cell death in vivo. To perform these experiments we modified our experimental timetable to include an additional preliminary task: the development of the development of estrogen sensitive clonal cell lines expressing enhanced Green Fluorescence Protein (GFP). We have therefore developed a number of MCF-7^{GFP} sublines, and have compared the effects of anti-estrogens on these breast cancer cell lines to ensure

*that the transfected cells retained their normal estrogen dependence and their responsiveness to anti-estrogens.[status completed]. These cell lines are currently being analyzed in parallel with the parental MCF-7 cells, we are isolating MCF-7^{GFP} that are also invasive, and we are characterizing the sensitivity of these fluorescent sublines to anti-estrogens and TNF α . We have identified several MCF-7^{GFP} sublines that has the same characteristics as the parental line so that we can perform the in vitro studies proposed in specific aim 3, using thoroughly tested, appropriate cell lines in these latter experiments. We are currently testing these cell lines in parallel with the parental MCF-7 cells and are trying to isolated MCF-7^{GFP}/_{inv}^{ae} cell lines comparable to those we have derived from the parental line [status: partially complete]. Estimated revised time frame: These experiments should be completed between months 36-40. This timeline extends beyond the 3 year funding period, but as we have pointed out in our initial description of specific aim 3 and the progress report, we understand that funding for the *in vivo* studies which would be initiated after month 36, has not been secured, and is not guaranteed.*

Note these time estimates are made on the basis that the grant period is now 36 months, and that the clock did not stop during the hiatus caused by our move from the Adirondack Biomedical Research Institute.

Effect of Move on the Project:

The transfer of our laboratory from the Adirondack Biomedical Research Institute (formerly the W. Alton Jones Cell Science Center) in Lake Placid to the Department of Biological Sciences at the University of Notre Dame, and the delay in transferring the grant to the new institution resulted in an 8 month hiatus. This coupled with the no-cost extension from the university, now puts us on target to complete the research in June, 2000. This effectively puts the project, which was originally planned as a two year program, in synchrony with the three year grants awarded by the DAMD in 1997. The data presented in this report will be presented at the Era of Hope Meeting in Atlanta Georgia in June 2000.

Other than the downtime, we have not experienced any adverse effects on the project resulting from the move. We have re-established each of the cell lines in culture since we moved and have demonstrated that the parental MCF-7 and the MCF-7^{GFP} cell lines have not been affected by the move. The facilities available in our new laboratories, which were constructed to our specifications, are considerably superior to the facilities we left in Lake Placid. A number of new instruments including superior microscopes and electronic image documentation and analysis for microscopy and gel electrophoresis (Western and RT-PCR) will significantly improve our throughput, making it possible to catch up for lost time.

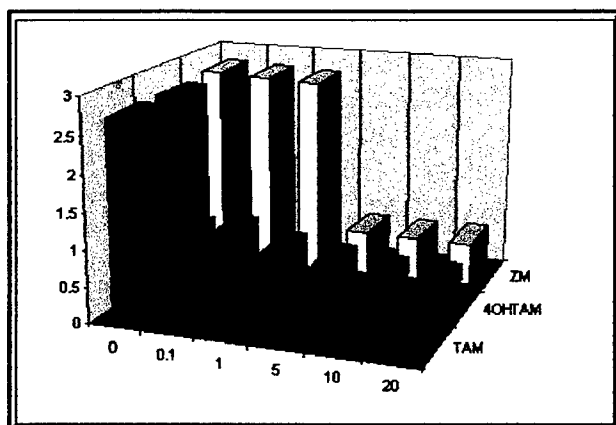


Figure 1

Dose response of MCF-7WT cells to various anti-estrogens. The dose range was from 0.1 to 20 μ M. The cells were plated in phenol red free α -MEM, with 10% FCS and treated with the anti-estrogens for 72 h. The surviving cell numbers were determined by crystal violet staining. Results represent mean of 3 independent experiments.

several anti-estrogens to TNF α on MCF-7^{WT} cells. We have established that the anti-estrogens induce cell cycle arrest and apoptosis of MCF-7^{WT} cells in a dose manner (Fig. 1). These data demonstrate that the MCF-7^{WT} display differential sensitivities to the individual anti-estrogens. Of the anti-estrogens tamoxifen and 4-hydroxytamoxifen appear to be considerably more potent (with an LD₅₀ of approximately 0.1 μ M), than the pure anti-estrogen, ZM 182, 780 (LD₅₀ of 2 μ M). Time course analysis of the effects of selected

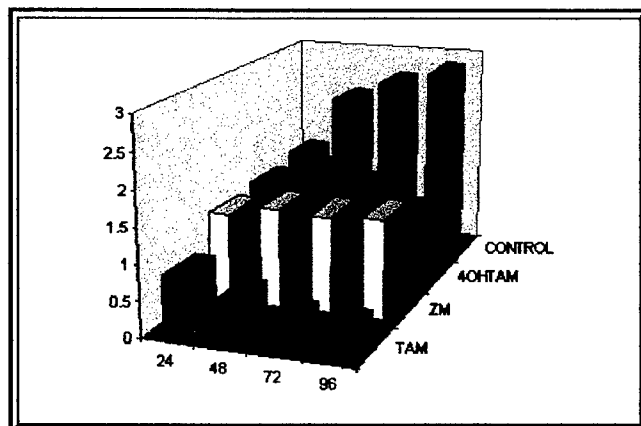


Figure 2

Time course of effects of various anti-estrogens on MCF-7^{WT} cells. Equal numbers of cells were plated in α -MEM and treated with 1 μ M tamoxifen, 4-hydroxytamoxifen or 10 μ M ZM 182,780. The cell numbers were determined by crystal violet at the indicated times. Results are the mean of 3 independent experiments

[This page contains unpublished data]

In the original application there were two specific aims:

Specific Aim 1. *To characterize the ability of anti-estrogens to induce apoptosis and an invasive phenotype in a sub-population of cells in vitro.*

Specific Aim 2. *To compare the sensitivity of MCF-7^{ae}_{inv} sublines to other agents that induce apoptosis and determine if multiple agents can act synergistically to induce apoptosis.*

Progress since Re-initiation of the Grant

We have compared the effects of several anti-estrogens to TNF α on MCF-7^{WT} cells. We have established that the anti-estrogens induce cell cycle arrest and apoptosis of MCF-7^{WT} cells in a dose manner (Fig. 1). These data demonstrate that the MCF-7^{WT} display differential sensitivities to the individual anti-estrogens. Of the anti-estrogens tamoxifen and 4-hydroxytamoxifen appear to be considerably more potent (with an LD₅₀ of approximately 0.1 μ M), than the pure anti-estrogen, ZM 182, 780 (LD₅₀ of 2 μ M). Time course analysis of the effects of selected doses of each anti-estrogen demonstrates that 1 μ M tamoxifen induces a clear reduction in cell number, while 1 μ M 4-hydroxytamoxifen and 10 μ M ZM 182,780 block the increase in cell numbers seen in the control, untreated cells. Flow cytometry studies have shown that the effects of all three anti-estrogens is due to a combination of cell cycle arrest and apoptosis (data not shown). Regardless of the dose and time of treatment it is important to recognize that a significant number of cells survive the anti-estrogen treatments.

We have now demonstrated that

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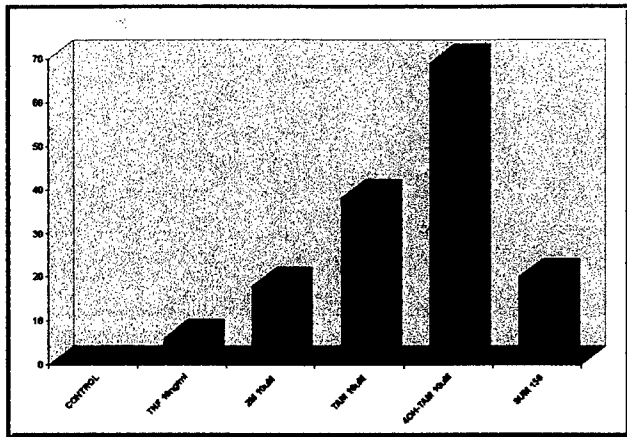


Figure 3:

Induction of an invasive phenotype in MCF-7^{WT} cells treated with TNF α and various anti-estrogens. Equally numbers of MCF-7^{WT} cells were plated in each well, and treated with TNF α , ZM 182, 780 tamoxifen or 4-OH tamoxifen for 72 h. Surviving cells were collected and equal numbers were plated on 8 μ membranes coated with Matrigel. Cell numbers in the lower chambers were determined 48 h later by crystal violet. The data were compared to the level of invasion using a known metastatic cell line (SUM 159T cells).

a sub population of MCF-7^{WT} that survive anti-estrogen therapy acquire an invasive phenotype after treatment. In preliminary experiments we have established the appropriate parameters for measuring the ability of the MCF-7^{WT} and MCF-7^{GFP} cells to invade through Matrigel in a modified Boyden chamber assay, by monitoring cell number (measured by crystal violet staining) or intrinsic GFP fluorescence as they pass through the Matrigel and membrane of the chamber before and after treatment with anti-estrogens. As shown in Figure 3, MCF-7^{WT} cells do not invade through 8 μ invasion filters coated with a thick layer of Matrigel in the absence of the anti-estrogens. However after treatment with tamoxifen or 4OH-tamoxifen, a sub population of the surviving cells acquire an invasive phenotype. Treatment of the MCF-7^{WT} cells with TNF α or ZM 182,780

(which also induce cell death), does not induce an invasive phenotype in the surviving cells. It is clear that treatment with tamoxifen or 4-OH tamoxifen increases the invasive potential of the MCF-7 cells to a level that is equal to or exceeds that of the SUM159T cells, a breast cancer cell line that we have shown is invasive *in vitro* and metastatic *in vivo*. As Figure 4 demonstrates the acquisition of the invasive phenotype is enhanced by the chemotactic attractants in fibroblast-conditioned medium (presumably growth factors such as basic FGF and FGF-9), but the invasive cells do not require the presence of the conditioned medium to manifest the phenotype.

These studies confirm that a small sub-population of cells that survive anti-estrogen therapy become invasive. Preliminary data, based on RT-PCR, demonstrate that anti-estrogen treatment of these sublimes induces the synthesis of several extracellular proteases (notably cathepsin B) while down-regulating the synthesis of at least one inhibitor of the matrix metalloproteases (TIMP-1). While these data clearly offer support

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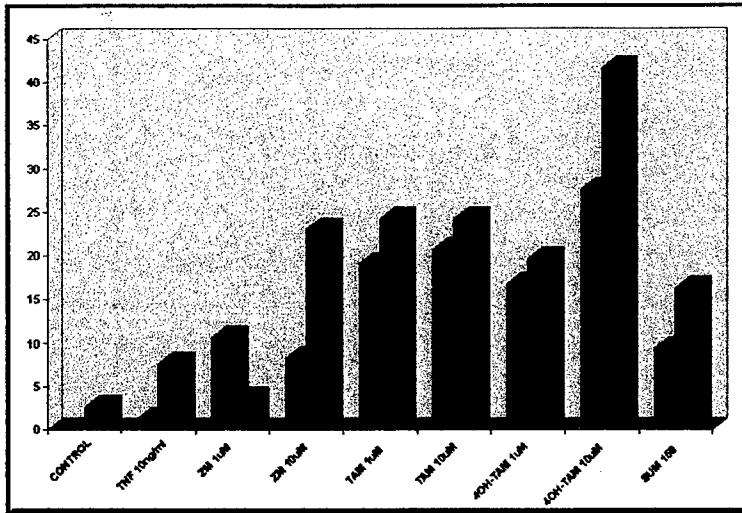


Figure 4

Effect of fibroblast-conditioned medium in the acquisition of the invasive phenotype. MCF-7^{WT} cells were grown, and treated with the indicated anti-estrogens as described in figure 3. Equal numbers of cells were plated on 8μ membranes coated with Matrigel. The lower chamber contained either α-MEM (blue) or fibroblast conditioned medium (purple). Cell numbers in the lower chambers were determined 48 h later by crystal violet. The data were compared to the level of invasion using a known metastatic cell line (SUM 159T cells).

to our hypothesis, they need to be repeated under rigorous experimental conditions to confirm this observation and to determine which other extracellular proteases (and acid hydrolases) are induced after treatment with anti-estrogens. We will also determine which inhibitors of matrix metalloproteases and cathepsins are simultaneously down regulated.

Prior to the initiation of the research we were asked to describe how we would pursue this work *in vivo* as part of a follow up study. At that time we added specific aim 3 to the research program, with the understanding that this specific aim was outside the scope of the two funding period.

Specific Aim 3: *To determine whether treatment with anti-estrogens or other apoptosis inducing reagents (such as vitamin D analogs) alters the rate and/or sites of metastasis and whether invasive cells generated by treatment with one reagent are sensitive to treatment with other reagents that induce cell death in vivo.*

While specific aim 3 was clearly not part of the original application, our proposal to use MCF-7 cells transfected with GFP (green fluorescent protein) to monitor invasive cells both *in vitro* and *in vivo* suggested to us that we would be well advised to create these MCF-7^{GFP} sublines before proceeding to examine the effects of anti-estrogens on these breast cancer cell lines to ensure that the transfected cells retained their normal estrogen dependence and their responsiveness to anti-estrogens.

[This page contains unpublished data]

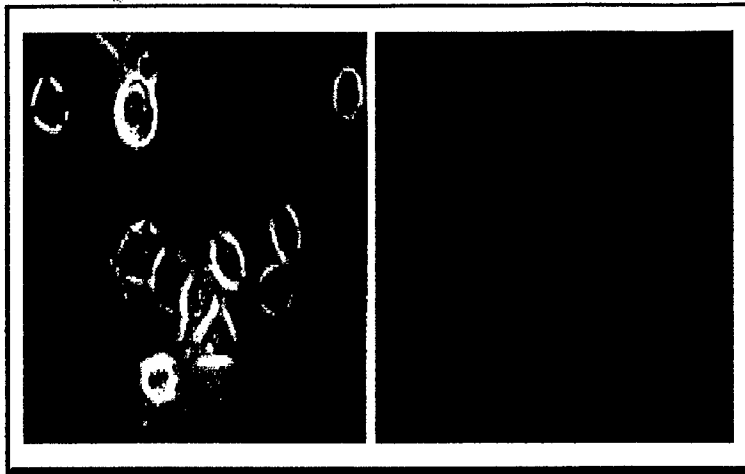


Figure 5: Phase contrast (left panel) and fluorescence microscopy (right panel) of MCF-7 cells transfected with a plasmid expressing green fluorescent protein (GFP). MCF-7^{GFP} cells were grown for 72 h in serum free defined medium prior to photography.

then sorted on a Becton-Dickinson fluorescence activated cell sorter into 96-well culture plates using the autosort facility of the machine. The cells in individual wells were clonally expanded to produce a series of high, medium and low GFP-expressing sublines by growth in G418. A total of 20 sublines have been stabilized, characterized and frozen in liquid nitrogen for further use. Phase contract and fluorescence photomicrographs of one of these sublines is shown in Figure 5, demonstrating that virtually all of the cells within the selected subline express GFP at approximately the same level.

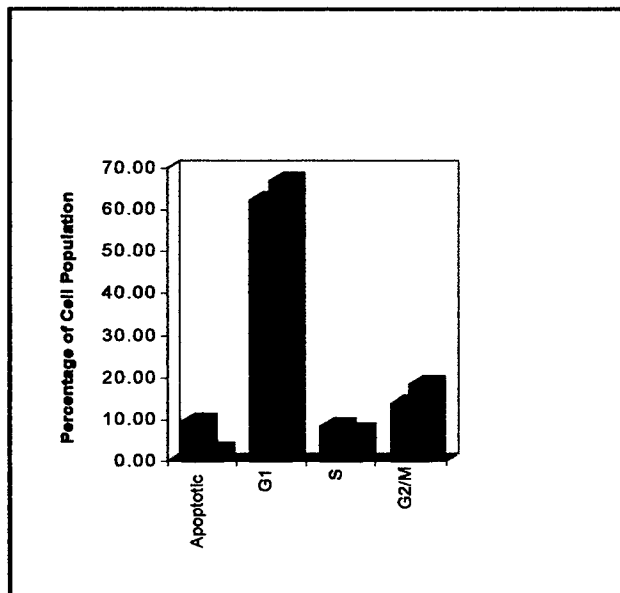


Figure 6
Comparison of cell cycle kinetics between parental MCF-7^{WT} cells (purple) and MCF-7^{GFP} (green). Cells were stained with propidium iodide and analyzed on a Coulter Epics flowcytometer.

In our initial experiments we have established that transfection of the MCF-7 cells with the GFP construct has not significantly affected the cell cycle kinetics (Fig. 6). Both MCF-7^{WT} and MCF-7^{GFP} have essentially the same proportion in each phase of the cell cycle, suggesting that the GFP transfection has not altered the cell cycle kinetics. We have also compared the sensitivity of these sublines to TNF- α with that of the

parental MCF-7 wild type cells (MCF-7^{WT}). Without exception, the MCF-7^{GFP}, retain their

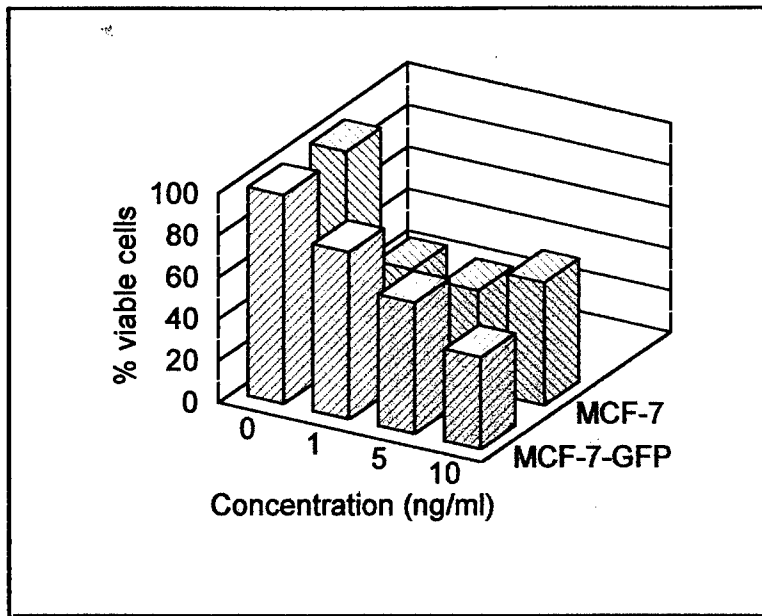


Figure 7: Comparison of sensitivity of MCF-7^{GFP} and parental MCF-7^{WT} to increasing doses of TNF- α . The two cell lines were grown for 4 days and then treated with increasing doses of TNF- α . After 72 h the number of surviving cells was estimated using crystal violet, and compared to untreated controls. Results are the mean of three independent experiments.

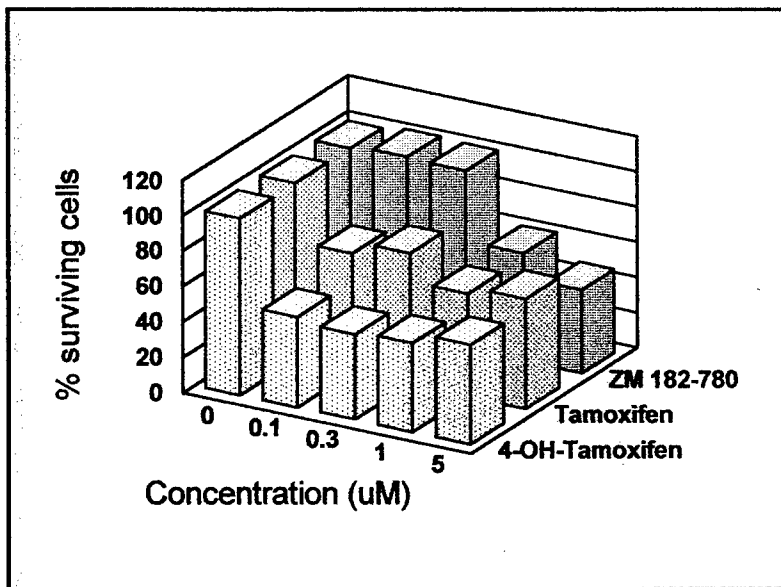


Figure 8: Dose response of MCF-7^{GFP} cells to ZM182,780, tamoxifen or 4-hydroxytamoxifen.

MCF-7^{GFP} cells were incubated with each of the indicated anti-estrogens for 72h. The cells were harvested and the cell viability was assessed using crystal violet and compared to untreated cells incubated for the same length of time. Results are average of two independent experiments.

sensitivity to TNF- α and demonstrate a very similar dose response to the drug as the parental wild type MCF-7 cells, suggesting that the expression of GFP in these cells does not influence the ability of the transfected cells to undergo apoptosis after the appropriate stimulation (Fig. 8).

Most, but not all of the transfected sublines have also retained their sensitivity to anti-estrogens and induce cell death (as monitored by DNA fragmentation (TUNEL positivity) and cell viability as monitored by crystal violet or flow cytometry. The relative sensitivity of the subline shown in Fig 1 and 2, to ZM182,780, tamoxifen and 4-hydroxytamoxifen is shown in Fig 3. The results demonstrate that this particular subline retains its sensitivity to all three anti-estrogens, and like the parental MCF-7 cell line appears to be sensitive to the pure anti-estrogen ZM 182,780 only at higher concentrations ($> 1 \mu\text{M}$), even though the overall rate of cell death appears to be of the same order of magnitude (approximately 40% of the cells appear to survive anti-estrogen therapy for 72h). Most of the sublines display similar sensitivities to the individual anti-estrogens although we have not yet

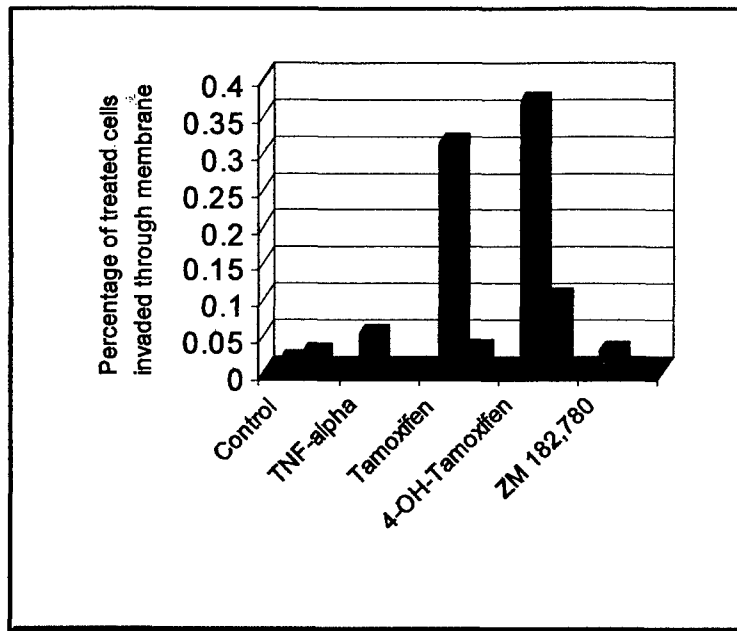


Figure 9 Induction of the invasive phenotype in MCF-7^{WT} (purple) and MCF-7^{GFP} (green) cells by anti-estrogens.

determined whether the (minor) variations we see are reproducible or biologically significant.

Importantly, both tamoxifen and 4OH tamoxifen induce an invasive phenotype in the MCF-7^{GFP} as they do with the MCF-7^{WT} cells (Fig. 8), suggesting that these cells are suitable for the *in vivo* studies outlined in specific aim 3.

In summary, the experiments required to complete Specific Aims 1 and 2 will be completed within the next 6-8 months, and the initial experiments required for the initiation and completion of

specific aim 3 have been completed. It is our intention to apply for a new grant from DAMD for the studies outlined in specific aim 3 in the next competition.



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